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Calcium Signalling: More Messengers, More Channels, More Complexity

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Recent studies have expanded the number of channel types and messengers that lead to Ca^{2+} signals within cells. Furthermore, we are beginning to understand the complex interplay between different sources of Ca^{2+}.

Cytosolic Ca^{2+} is a focal point of many signal transduction pathways and regulates cellular activities ranging from fertilisation to cell death [1]. It is known that cytosolic Ca^{2+} increases can be temporally and spatially complex. Depending on cell type and the nature of stimulation, Ca^{2+} signals can be transient or oscillatory, and can occur as localised or global events [2]. Given this diversity of cellular Ca^{2+} signals, it is not surprising that there are multiple messengers and channels through which Ca^{2+} elevations can occur.

The established intracellular messengers that increase cytosolic Ca^{2+} include inositol 1,4,5-trisphosphate (IP_3), cyclic adenosine 5′-diphosphoribose (cADPR), nitric oxide (NO), H_2O_2/O_2^-, nicotinic acid adenine dinucleotide phosphate (NAADP), diacylglycerol, arachidonic acid, sphingosine, sphingosine-1-phosphate and Ca^{2+} itself. Some of these messengers act on intracellular Ca^{2+} release channels, others on Ca^{2+} entry channels, whilst a couple act on both release and entry. From the specificities of the Ca^{2+}-releasing messengers we know that there must be several different types of intracellular Ca^{2+} release channel, although at present only IP_3 receptors and ryanodine receptors have been characterised in detail.

A range of Ca^{2+} influx channels have been established for some time. In addition to their activation by some of the messengers listed above, Ca^{2+} influx channels are activated by stimuli including membrane depolarisation, stretch, noxious stimuli, extracellular agonists and depletion of intracellular stores [1]. Recent studies have expanded the numbers of Ca^{2+}-increasing messengers and channels yet further. In addition, it is becoming evident that different Ca^{2+} signalling pathways can interact to control the source and characteristics of cytosolic Ca^{2+} signals.

A critical feature of both IP_3 receptors and ryanodine receptors is that Ca^{2+} itself regulates their opening. Their sensitivity to cytosolic Ca^{2+} allows them to act as Ca^{2+}-induced Ca^{2+} release (CICR) channels which promote the amplification of smaller trigger events. For IP_3 receptors, it was thought that the binding of IP_3 was obligatory for channel opening. In a recent study, however, Foskett and colleagues [3] demonstrated that a protein could supplant the need for IP_3. These authors found that a family of neuronally expressed proteins known as Ca^{2+}-binding proteins can engage IP_3 receptors and promote channel opening. These proteins have Ca^{2+}-binding 'EF-hand' motifs and belong to the superfamily of EF-hand proteins. The interaction of Ca^{2+}-binding proteins with IP_3 receptors is positively regulated by Ca^{2+} and not dependent upon the presence of IP_3. In cells expressing Ca^{2+}-binding proteins, IP_3 receptors may therefore be activated independently of IP_3 production. It is known that accessory proteins, in addition to factors such as pH, thiol modification and phosphorylation, can regulate IP_3 receptors. But Ca^{2+}-binding proteins are so far the only entities that can fully open IP_3 receptors in the complete absence of IP_3.

Depletion of intracellular Ca^{2+} stores by activation of IP_3 or ryanodine receptors switches on a Ca^{2+} influx pathway through store-operated channels (SOCs). The mechanism underlying SOC activation, and the identity of the channels involved, are unclear. It was thought that SOCs provide the main route for Ca^{2+} entry into non-electrically excitable cells. However, accumulating evidence suggests that intracellular messengers can activate Ca^{2+} influx during physiological stimulation. When IP_3 is produced from phosphoinositide hydrolysis, there is a concomitant production of diacylglycerol (DAG). Unlike the water soluble IP_3, DAG stays in the plane of the plasma membrane where it can activate protein kinase C (PKC) or be metabolised in various ways. PKC and DAG have both been shown to cause Ca^{2+} influx distinct from SOC. Furthermore, other messengers resulting from DAG metabolism, including arachidonic acid and leukotrienes, activate non-SOC (NSOC) Ca^{2+} influx.

It is interesting that different cell types are responsive to alternative messengers on the DAG/PKC/arachidonate/leukotriene pathway. How and why cells use different metabolites to regulate NSOC entry is not known. The suggestion that such messengers control Ca^{2+} entry is not new, but recent studies have begun to determine their contribution to cellular responses. A significant observation with regard to arachidonate-activated Ca^{2+} influx, first noted by Shuttleworth and colleagues [4], is that it is activated by lower concentrations of hormonal stimulation than are required to evoke SOC. It is generally thought that the response to the lowest concentration of agonist represents a physiological situation. Arachidonate-induced Ca^{2+} entry may therefore be the principal influx route during physiological levels of cell stimulation.

In the system studied by Shuttleworth and colleagues, Ca^{2+} entering through SOC channels inhibited arachidonate-induced Ca^{2+} entry. The significance of this is that the SOC and NSOC pathways do not transport Ca^{2+} simultaneously (unless cytosolic Ca^{2+} is buffered at $\leq$100 nM). NSOC is responsible for Ca^{2+} entry at low stimulus concentrations, but this switches to SOC as the stimulus level increases and endoplasmic

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reticulum (ER) Ca^{2+} stores are depleted. A similar switch between NSOC and SOC has been observed by Taylor and colleagues [5]. In their system, NSOC and SOC were reciprocally regulated by arachidonate itself. During stimulation of cells with an agonist that activates phosphoinositide hydrolysis, the arachidonate produced both activated NSOC and inhibited SOC. Only after the removal of agonist and subsequent metabolism of arachidonate was SOC evident. The data from the Shuttleworth [4] and Taylor labs [5] both provide evidence for switching between mutually exclusive pathways for Ca^{2+} influx in the same cell.

The route for arachidonate production is not the same in these two studies. In Shuttleworth's human embryonic kidney cells, arachidonate is produced by a G-protein regulated phospholipase A_2 [4]. Whilst in the A7r5 smooth muscle cells studied by Taylor's group [5], arachidonate results from metabolism of DAG. Indeed, there are other pathways leading to arachidonate production in cells, which may also activate NSOC. The critical point is that, by using a metabolite such as arachidonate, Ca^{2+} influx is directly tied to cell stimulation. Why, then, have multiple pathways for Ca^{2+} influx? One obvious answer is that different Ca^{2+} influx routes may be spatially segregated and activate distinct Ca^{2+}-sensitive processes [2]. In addition, as restoration of Ca^{2+} within the ER is necessary for protein synthesis and folding, SOC may plausibly act as a 'pressure valve' to allow replenishment of lumenal Ca^{2+}, but perhaps only after substantial depletion.

At present, the best molecular candidates for SOC and NSOC channels are the TRP proteins (so-called because of their homology with the transient receptor potential protein that underlies phototransduction in *Drosophila*). The TRP superfamily has been subdivided into multiple subfamilies on the basis of sequence similarity [6]. In the case of SOC, much attention has been focussed on the canonical TRP (TRPC) subfamily. Despite considerable effort, it is unclear exactly which of the seven TRPC isoforms are the molecular constituents of endogenous SOCs. It was recently suggested that a channel belonging to the TRPV subfamily, CaT1 (TRPV6), could be a candidate for a form of SOC known as I_{CRAC} (Ca^{2+}-release activated current) [7]. However, the correlation between CaT1 and I_{CRAC} has been disputed [8].

Although the mechanism underlying SOC is not known, some recent studies of TRP isoforms have added to the list of stimuli that evoke Ca^{2+} influx through these channels. For example, some TRP proteins are activated by environmental cues such as temperature changes. Indeed, different TRP isoforms (members of the TRPV and TRPM subfamilies) are activated across a range of temperatures from ~5 to >52°C (for example [9]). Interestingly, the TRPs that detect cold or heat can be expressed on the same sensory neurons, and a Ca^{2+} signal is a common response to these different stimuli. TRPM7 appears to be regulated by intracellular Mg.ATP [10]. Physiological levels of Mg.ATP, in the millimolar range, suppress this channel, but if ATP levels drop then Ca^{2+} can enter the cell. Another related TRPM member, TRPM2, is activated by oxidation [11] and ADP ribose (a breakdown product of the Ca^{2+}-mobilising messenger cADPR [12]), and negatively regulated by intracellular ATP levels [13].

The regulation of cADPR and ADPR levels inside cells is not well understood, although a few different enzymatic activities have been suggested to underlie the conversion of β-NAD into these messengers. One interesting moiety is CD38, a widely-expressed glycoprotein that spans the plasma membrane and some internal organelles [14]. CD38 has been suggested both to produce cADPR and NAADP and to transport them into cells. This enzyme is highly expressed on hematopoietic cells and its significance in immune responses was recently demonstrated using CD38 knock-out mice [15]. These mice were more susceptible to lethal bacterial infection of the lungs, seemingly because of a lack of neutrophil chemotaxis. Analysis of the Ca^{2+} signals in the neutrophils of the $CD38^{-/-}$ mice revealed that the responses were smaller and less prolonged than in the wild-type animals.

The fact that Ca^{2+} signals in the neutrophils of the $CD38^{-/-}$ mice were reduced, but not completely absent, suggests that the responses of wild-type cells reflect the simultaneous activation of multiple Ca^{2+} signalling pathways. Indeed, as described above for SOC and NSOC Ca^{2+}entry, the cooperation or switching between different sources is becoming a prevalent theme in Ca^{2+} signalling. An example of the complex interplay between different Ca^{2+} release channels was recently demonstrated using pancreatic acinar cells [16]. Essentially, Petersen and colleagues [16] demonstrated that by using combinations of different Ca^{2+}-mobilising messengers — IP_3, cADPR and NAADP — they could reconstruct the local and global Ca^{2+} signals generated by physiological stimuli. The data suggest a hierarchy of channel communication, with NAADP perhaps being the base effector, subsequently supplying Ca^{2+} to enhance IP_3 and cADPR responses.

Mast cells also appear to concurrently utilise different messengers — in this case IP_3 and sphingosine-1-phosphate to generate the Ca^{2+} signals that underlie the synthesis and release of inflammatory mediators [17]. Essentially, the antigen receptors on these cells trigger multiple signalling pathways. One of these is phosphoinositide hydrolysis, leading to IP_3 production. Another is the stimulation of phospholipase D, which hydrolyses phosphatidylcholine into phosphatidic acid and choline. It has been suggested that phosphatidic acid can activate a kinase that phosphorylates sphingosine into sphingosine-1-phosphate. The dual activation of these pathways leads to a Ca^{2+} signal with a rapid peak (sphingosine-1-phosphate dependent) and a sustained plateau (IP_3 dependent) [17].

Exactly how sphingosine-1-phosphate causes Ca^{2+} release from intracellular stores is unclear. Until recently, the best candidate for the sphingosine-1-phosphate receptor was a protein known as 'sphingolipid Ca^{2+}-release mediating protein of endoplasmic reticulum' (SCaMPER). It had been proposed that this protein formed a widely occurring channel responsive to sphingosine-1-phosphate and sphingosylphosphorylcholine. A recent re-investigation of SCaMPER found

that there was little correlation between its intracellular location and that of known intracellular Ca^{2+} stores [18]. Furthermore, expression of SCaMPER was found not to confer sensitivity to sphingolipids nor to affect Ca^{2+} homeostasis, but could lead to cell death.

Although the possibility that SCaMPER provides an intracellular Ca^{2+} release mechanism is fading, the evidence implicating another type of channel is growing. Autosomal dominant polycystic kidney disease (ADPKD) is the most common genetic cause of renal failure. It is caused by mutations in proteins called polycystins, which are members of the TRPP subfamily that can function as Ca^{2+} channels. Although there are five polycystins, mutations in two of the most widely expressed family members, polycystin-1 and polycystin-2 (PC-2), are particularly important with respect to ADPKD. A recent study [19] has added to the weight of evidence that PC-2 acts as an intracellular Ca^{2+} release channel. Divalent cation currents were observed when ER vesicles from cells expressing PC-2 were fused into bilayers. Furthermore, responses of intact cells to Ca^{2+}-mobilising hormones were increased significantly following exogenous expression of PC-2. PC-2 activation appears to be triggered by Ca^{2+}. PC-2 has a functional EF-hand that binds Ca^{2+}, just as described above for the Ca^{2+}-binding proteins. The opening of the PC-2 channel in bilayers was increased as Ca^{2+} on the cytosolic face was increased, and truncation of the carboxy-terminal tail of the protein where the EF-hand is located abrogated this effect. Given their wide spread expression, polycystins may comprise an important family of CICR channels.

The Ca^{2+} signalling field is forging ahead on many fronts. We have a better understanding of how different messengers and channels generate Ca^{2+} signals, and their roles in defined physiological and pathological processes. The multiplicity of messengers and channels does not reflect redundancy, but rather points to a complex Ca^{2+} signalling 'toolkit' tuned to individual cellular requirements [1]. Clearly, Ca^{2+} will be causing excitement for many years to come.

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